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Report

Fitness-Associated Sexual Reproduction in a Filamentous Fungus

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Summary

Sex is a long-standing evolutionary enigma. Although the majority of eukaryotes reproduce sexually at least sometimes [1–3], the evolution of sex from an asexual ancestor has been difficult to explain because it requires sexually reproducing lineages to overcome the manifold costs of sex, including the destruction of favorable gene combinations created by selection [4, 5]. Conditions for the evolution of sex are much broader if individuals can reproduce either sexually or asexually (i.e., facultative sex) and allocate disproportionately more resources to sex when their fitness is low (fitness-associated-sex or FAS [6–10]). Although facultatively sexual organisms have been shown to engage in more sex when stressed [11], direct evidence for FAS is lacking. We provide evidence using 53 genotypes of the filamentous fungus *Aspergillus nidulans* in a reciprocal transplant experiment across three environments. Different genotypes achieved highest fitness in different environments and genotypes invested relatively more in sex in environments in which their fitness was lower, showing that allocation to sexual reproduction is a function of how well-adapted a genotype is to its environment. FAS in *A. nidulans* is unlikely to have evolved as a strategy to resist or avoid stress because asexual spores are more dispersive and equally resistant [12, 13].

Results and Discussion

Facultative sexuality is of particular interest in understanding the origins and maintenance of sex because it is both common [10, 11] and thought to represent the ancestral state of sexual reproduction [14]. Recent theory suggests that facultative sex can readily evolve from an ancestral asexual state if individuals allocate disproportionately more resources to sexual reproduction when their fitness is low (termed fitness-associated sex, FAS). The evolutionary advantage to an allele causing FAS arises because it tends to remain on, and preserve, the good genetic backgrounds of high fitness individuals, yet can segregate out of comparatively poor genetic backgrounds of low fitness individuals [9], a phenomenon known as the "abandon-ship" mechanism. The conditions for the evolution of FAS may be further broadened if the costs of sex can be reduced [9], as may happen if sexual reproduction—that takes substantially more time to complete than asexual reproduction—occurs preferentially when asexual growth rates are low [15, 16].

Facultative sexual species often engage in sexual reproduction in response to environmental stress or degradation, suggesting that individuals in poor condition are more likely to undergo sex [11]. It is also well known, for instance, that the protocol for inducing a sexual cycle in eukaryotic model microorganisms, such as the unicellular chlorophyte *Chlamydomonas reinhardtii* and yeast, involve starving genotypes for essential nutrients like nitrogen [17–19]. Although such observations are consistent with FAS, they do not constitute direct evidence for it because the possibility cannot be excluded that a degraded or stressful environment acts as a nonspecific trigger to initiate sexual reproduction. Models for the evolution of FAS predict, by contrast, that the level of investment in sexual reproduction depends exclusively on fitness as a measure of the match between an organism and its environment, and not some environmental factor common to all genotypes. Specifically, under FAS a genotype is expected to invest relatively more in sexual reproduction in an environment to which it is maladapted and, conversely, relatively less in an environment to which it is well adapted [4]. A direct demonstration of FAS, therefore, requires showing that the level of sexual investment depends solely on a genotype's fitness, as opposed to being a general property of the environment or the genotype itself. To the best of our knowledge, such evidence is currently lacking.

As a direct test of FAS in the facultatively sexual filamentous fungus, *Aspergillus nidulans*, we conducted a reciprocal transplant experiment involving 53 genotypes and three environments (see Experimental Procedures). *A. nidulans* grows as a mycelium composed of a dense network of hyphae. It reproduces asexually through nuclei in mycelium and conidiospores, and sexually through fruiting bodies (cleistothecia) containing ascospores (Figure 1) [20, 21]. Forty-five of the genotypes used here were derived from a common antifungal (fludioxonil) resistant ancestor and had independently evolved asexually for 800 generations under fungicide-free conditions [22]. Although all populations adapted to the selection environment, they did so by different genetic routes: among populations at the end of the experiment we observed variable numbers of beneficial mutations fixed, substantial variation in final fitness, and significant genotype-by-environment interaction when these populations were tested under novel conditions. Sexual crosses of evolved genotypes with the ancestor also showed the fixation of multiple mutations in the evolved genotypes [22]. Thus although we do not know the DNA sequence identity of each genotype, we can be confident that these constitute a range of distinct genotypes. The remaining eight genotypes were chosen arbitrarily from two stock centers (see Supplemental Information available online).

For each of the 53 genotypes, we measured fitness and investment in sexual reproduction in each of three environments (see Experimental Procedures). Fitness was assayed as mycelial growth rate (MGR) by measuring colony diameter after 5 days of growth [22, 23]. Sexual investment was quantified as the density of sexual fruiting bodies in a given area of mycelium [24]. The environments were composed of a standard rich medium (CM), and the same medium supplemented or not with one of two distinct fungicides (CMflu: fludioxonil;

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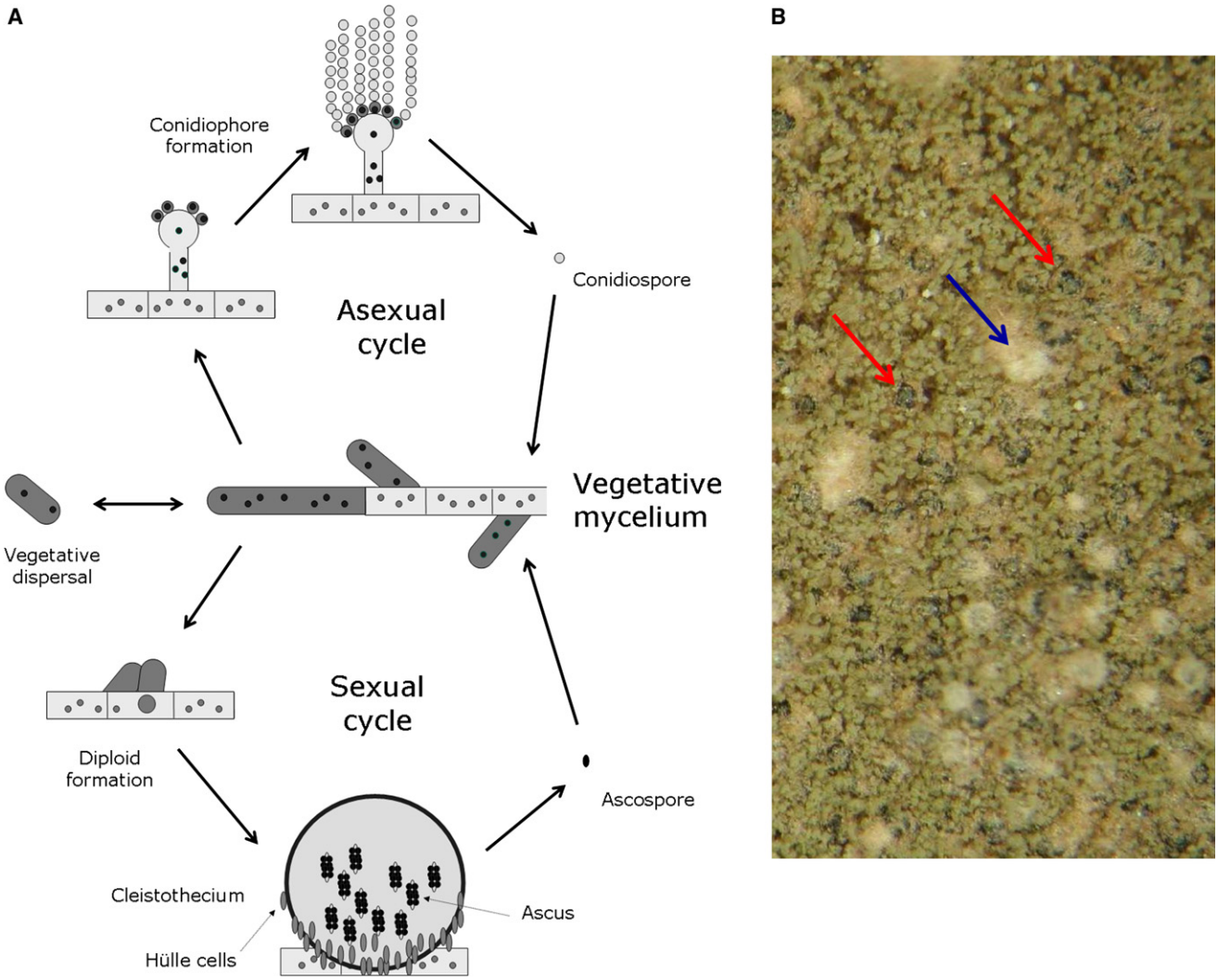

Figure 1. *Aspergillus nidulans*

(A) Life cycle. A single nucleus within either an asexually derived conidiospore, a sexually derived ascospore, or within vegetative mycelium, can give rise to the formation of a mature vegetative mycelium consisting of a network of hyphae. Mature hyphae are subdivided by septa into subapical cells (light gray), each containing around four nuclei, and mitotically active apical cells (dark gray). Twenty-four hours after the hyphae have first developed, some subapical cells differentiate into foot cells that give rise to stalks carrying a spore-head (*conidiophore*). Specialized cells on the spore-head branch off numerous asexual spores by repeated mitoses. A typical spore head will mature in around 24 hr after formation of the foot cell and will hold ~10,000–50,000 conidiospores. Around 5 days after the formation of the hyphae, some cells will initiate a sexual cycle for which two haploid nuclei fuse to form a diploid. These nuclei may be identical, one or both may carry one or more newly arisen mutations, or they may derive from different individuals if the mycelium was a heterokaryon from a previous vegetative fusion of mycelium with different genotypes. This diploid goes through a few rounds of mitotic division, followed by meiosis. A sexual fruiting body (*cleistothecium*) is formed with the support of Hülle cells that fall off after maturation. After ~6 days, the sexual fruiting body is mature and will typically contain 100,000–500,000 ascospores.

(B) Picture of mature mycelium viewed at approximately 10× magnification. The green carpet is formed by the many conidiophores carrying green spores. The larger black circles (indicated by red arrows) are sexual fruiting bodies. Some are not fully mature yet and still have the light brown Hülle cells around them (blue arrow).

CMacr: acriflavin). Fungicide levels were sufficient to reduce growth but not kill the genotypes, and the resulting fitness values spanned a more than three-fold range.

Across environments, average investment in sexual reproduction and population mean fitness were inversely correlated, and the same trend was observed among genotypes within each environment (Figure 2; linear regression slopes are significantly negative: CM, $F_{1,51} = 7.95$, $R^2 = 0.135$; $p = 0.007$; CMflu, $F_{1,51} = 29.5$, $R^2 = 0.367$, $p < 0.0001$; CMacr, $F_{1,51} = 9.67$, $R^2 = 0.159$, $p = 0.003$). These results are consistent with FAS and with previous observations from other facultative sexual organisms. However, such a result could also arise in the absence of FAS if genotypes vary in their average investment in sex and this investment trades-off with growth. Under this scenario, genotypes investing more in sex should do so consistently across environments. This was not the case. Rather, fitness depended on an interaction between genotypes and their environment ($F_{45,268} = 636$; $p < 0.0001$) and a substantial proportion of this interaction (89.2%) was caused by changes in the rank fitness of genotypes across environments (often termed "inconsistency" [25, 26]; Figure 3). These differences in rank-order among environments are significant

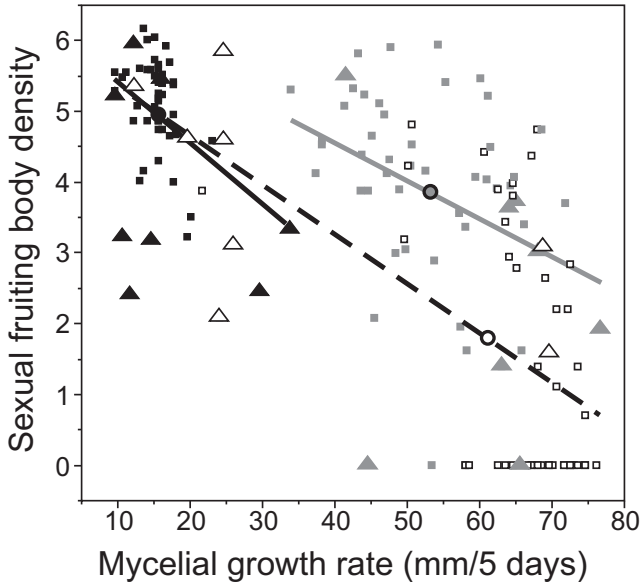

Figure 2. Investment in Sexual Reproduction

Sexual fruiting body density [*ln* (count + 1)] as a function of fitness (MGR) for the 45 evolved genotypes (squares) and the eight stock center genotypes (triangles) measured in each of three environments: CMacr (filled symbols, black line), CMflu (open symbols, dashed line) and CM (gray symbols and line). Average sexual investment declines as mean fitness increases among the environments (large circles) and this pattern holds among genotypes within each environment (regression lines).

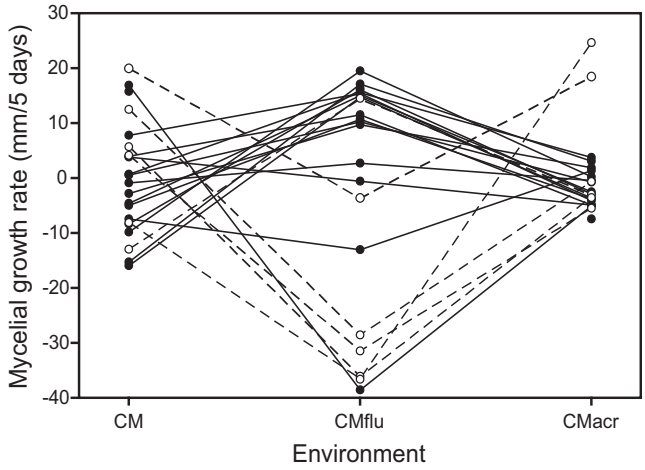

Figure 3. Fitness (MGR) of a Random Subset of 19 Genotypes across the Three Environments

Other genotypes were omitted for clarity. Growth rate is standardized to a mean of zero within each environment, removing any mean differences among environments. Solid lines are genotypes from the evolution experiment; dashed lines are stock center genotypes. Error bars (representing standard error) are not visible beyond the symbols.

(nonparametric ANOVA, $F_{45,268} = 234$; $p < 0.0001$), indicating that the environment in which highest fitness was achieved varied among genotypes. Notably, these results are qualitatively unchanged when the analyses are restricted to the stock center genotypes (81.7% inconsistency; nonparametric ANOVA, $F_{10,65} = 194$; $p < 0.0001$). Genotype-by-environment interactions of sufficient magnitude to generate changes in the rank order of fitness among environments are thus a general feature and not an unusual property of the genotypes from the selection experiment.

After removal of any confounding effects of environment (see Experimental Procedures), the residual investment in sexual reproduction of each genotype tended to decrease as its fitness increased (Figure 4A). This inverse relationship between fitness and sexual reproduction was significant across genotypes (Figure 4B; one-sample t test against an expected slope of zero: $t_{52} = -2.92$, $p = 0.003$), establishing FAS. The same trend is observed and approaches significance when we consider only the eight stock center genotypes ($t_7 = -1.80$, $p = 0.057$; $p = 0.039$ in a nonparametric signed-rank test), suggesting it does not reflect a degradation of sexual function caused by selection for asexual growth during experimental evolution.

How best to measure fitness is a controversial topic [27–29], most notably in laboratory studies where it is empirically intractable to include all components that interact to determine a genotype's total contribution to subsequent generations. Mycelial growth rate is the standard measure in filamentous fungi and strongly correlates with both total spore production and the number of nuclei present in a fungal colony [23, 24, 30, 31]. We have also shown previously a strong correlation between MGR and competitive ability via classic head-to-head multigeneration competition assays between the evolved strains used in the current study and a standard competitor strain [22]. Competitive ability is likely to be a good measure of lifetime fitness because it integrates both mycelial growth and conidiospore production across generations. An alternative fitness measure is provided by the total investment in nuclei, which we also determined (see Experimental Procedures). As with MGR, after removal of the main effects of environment, the residual investment in sex of a genotype declined with increasing total investment in reproductive nuclei and the pattern was significant across genotypes (one-sample t test against an expected slope of zero, $t_{49} = -2.07$, $p = 0.044$). Taken together, our results therefore appear to be robust to the fitness metric used.

Our results provide direct evidence for FAS and so lend support to the idea that it represents a feasible first step in the evolution of sexual reproduction. Facultative sexual organisms have been shown previously to engage in sex more often when stressed. Starvation, for example, induces sex in a variety of organisms [e.g., 6, 17, 19, 32, 33] and several species of plant have been shown to reproduce sexually more often under stressful conditions such as high density [34–37]. *Daphnia* also show an increased tendency to engage in sex when confronted with an epidemic of pathogenic bacteria [38], and sex has been shown to occur as a consequence of host immune response in the nematode *Strongyloides ratti* [39, 40]. In the absence of a full reciprocal transplant, however, these studies cannot separate genotypic and environmental effects.

Why the vast majority of eukaryotes reproduce sexually is an evolutionary paradox given the substantial costs of sex relative to asexual reproduction. In addition to the disruption of high fitness gene combinations built by selection [4, 5, 41], sex demands extra time and energy associated with meiosis and acquiring a mate, the latter of which may also expose individuals to increased risk of predation, injury, and disease. Reproducing sexually also transmits only 50% of an individual's genes to their offspring (as compared to 100% for asexual reproduction), generating a substantial two-fold cost

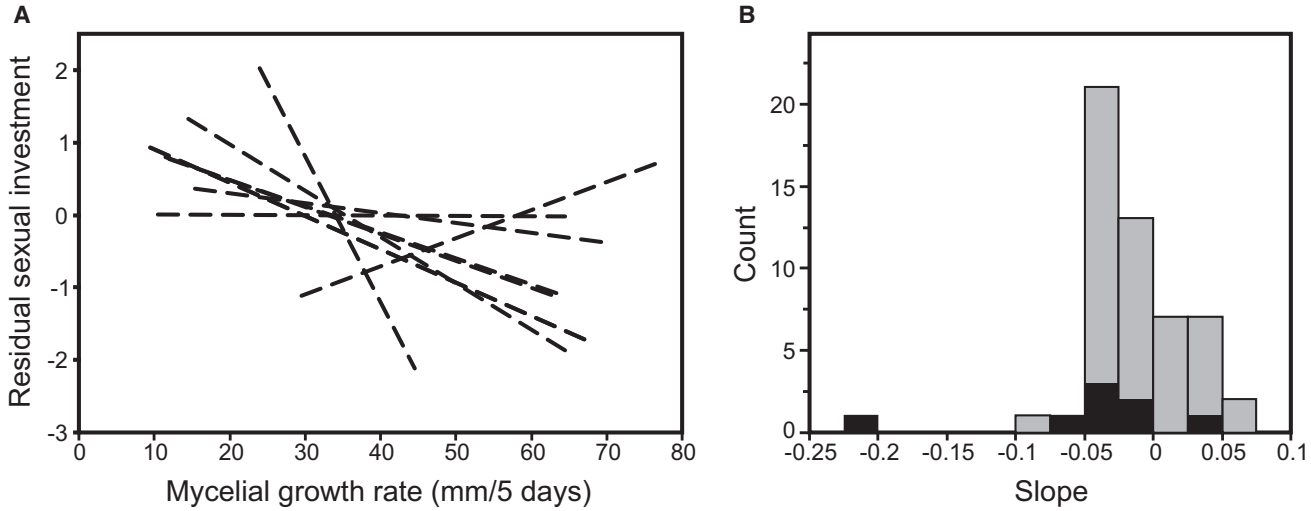

Figure 4. Fitness-Associated Sex

(A) Sexual investment as a function of fitness across the three environments. For clarity, only eight stock center genotypes are shown. Lines are linear regressions of the residual investment in sexual reproduction (after removal of the main effects of environment) against fitness (MGR) separately for each genotype (see Experimental Procedures).

(B) The frequency distribution of the slopes from the regressions in (A) for all 53 genotypes. Black bars depict stock center genotypes and gray bars show the genotypes from the evolution experiment.

in the absence of parental care [11]. Conditions for the evolution of sex are much broader if it occurs preferentially in low fitness individuals, and recent theory suggests that such FAS may evolve even in the face of substantial costs [9].

FAS is unlikely to have evolved in *A. nidulans* as a dispersal strategy in response to environmental stress or degradation. Unlike many taxa, asexual conidiospores represent an airborne phase in the life cycle and have higher dispersal rates than the sexually derived ascospores [12, 13]. Along similar lines, in many organisms sex involves the switch to a life stage that is more resistant of environmental stress, suggesting that sex could be a by-product of the evolution of stress resistance. In *A. nidulans*, however, no difference has been found in the ability of conidiospores versus ascospores to survive harsh conditions including drought, radiation, and high temperature [12].

Why FAS has evolved in *A. nidulans* is not known. Recent theory suggests that a modifier allele causing FAS would not only be strongly favored for purely selfish reasons, but might also increase population mean fitness [10]. To the extent that the relative costs of sexual reproduction can be reduced by restricting its lengthier process to times of slow growth [15, 16], the conditions for selection of a modifier allele causing FAS may be substantially broader [9]. However, this mechanism cannot by itself explain the origins of facultative sex because there is no mechanism to generate a fitness advantage to a sexually reproducing lineage.

Finally, the life cycle of *A. nidulans* (Figure 1A) may complicate the evolution of sex. The benefits of sex require the segregation and recombination that result from the fusion of genetically distinct nuclei (i.e., outcrossing). Although *A. nidulans* is homothallic, meaning that genetically identical nuclei can fuse to initiate a sexual cycle that results in selfed offspring, genetically distinct nuclei tend to fuse far more frequently than expected [42]. Observed recombination rates are high [43] and appear sufficient to achieve linkage equilibrium among natural isolates [44]. There is therefore little reason to believe that FAS could not evolve in this system. Key to evaluating the evolutionary origins of FAS in *A. nidulans* will therefore be experimental tests of whether individuals engaging in it can invade an asexual population [45]. The evolution of obligate sexuality, either from a facultatively sexual intermediate or de novo from asexuality, remains a major challenge for evolutionary biology.

Experimental Procedures

Fungal Strains

We used 53 genotypes of *Aspergillus nidulans*. Eight came from stock centers (Fungal Genetics Stock Center and Wageningen University; see Supplemental Information, Table S1, and Table S2). One of these strains (WG615) is resistant to the fungicide fludioxonil and this resistance confers an approximately 50% reduction in growth rate relative to the fungicide-sensitive ancestor in the absence of fludioxonil [22]. WG615 was used to found a selection experiment in which 120 replicate populations independently adapted to fungicide-free conditions for 800 generations [22]. Strains were grown as haploids on solid CM medium and were propagated every 5 days by serial transfer to fresh medium of a random sample of all nuclei present. For 60 populations, this transfer involved approximately 500 nuclei (small bottleneck), whereas for the other 60 populations it involved the transfer of approximately 50,000 nuclei (large bottleneck). The time between transfers was too short for sexual spores to develop [13]. Subsequent assays revealed that adaptation to these conditions was achieved through the substitution of one to three beneficial mutations without compromising fludioxonil resistance. Fitness of the evolved lines improved rapidly over the first 400 generations and typically remained relatively constant thereafter with different lines reaching different final fitnesses. From generation 800 of this selection experiment, 45 evolved genotypes were arbitrarily chosen for use in the current study: 27 from the small bottleneck treatment and 18 from the large bottleneck treatment.

Environments

Standard (9.5 cm) Petri dishes were filled with 30 ml of one of three different solid agar media: standard complete medium (CM; set at pH 5.8 and consisting of $NaNO_3$ 6.0 g/l; KH_2PO_4 1.5 g/l; $MgSO_4.7H_2O$ 0.5 g/l; NaCl 0.5 g/l; 0.1 ml of a saturated trace element solution containing $FeSO_4$, $ZnSO_4$, $MnCl_2$, and $CuSO_4$; tryptone 10 g/l; yeast extract 5 g/l; agar 10 g/l and

(added after autoclaving) glucose 4.0 g/l); CM with the fungicide fludioxonil 20 ppm (CMflu); and CM with the fungicide acriflavin 5 μg/ml (CMacr).

Fitness Assay

Strains were inoculated in the center of a Petri dish with 5 μl of dense spore suspension and grew as monokaryotic haploids. Colony diameter (MGR) was measured after 5 days of incubation at 37°C as the average of two replicate measurements taken in orthogonal directions.

Genotype × Environment Assay

In an assay separate from above, and with a random subset of 19 genotypes that included six from the stock centers (Table S1), MGR was measured in each environment with 5-fold replication. MGR was modeled as a linear function of environment, genotype, and their interaction in a two-factor crossed analysis of variance (ANOVA). Both main effects and their interaction were significant ($p < 0.0001$). The genotype × environment variance was subsequently partitioned into two components, one due to the variation in the environmental sensitivity of genotypes (also known as "responsiveness" [26] and "scale effects" [25]) and the other due to changes in rank (also termed "inconsistency" [25, 26]). The significance of the latter was evaluated with a nonparametric test in which a two-factor ANOVA was performed on the global (i.e., across environments) ranks of the observed fitness values [46].

Investment in Sexual Reproduction Assay

By previously described methods [24], 30 μl of the same dense spore suspension used for the fitness assays was spread on each of the three solid media (CM, CMflu, and CMacr). After 12 days of incubation, when sexual fruiting bodies have fully matured (Figure 1B), a digital photograph of each Petri dish was taken with a macroscope. From the pictures, we counted the number of sexual fruiting bodies in a standard area of 6.4 × 4.2 mm. (Because the colony inoculated onto each Petri dish derives from a single haploid genotype, the diploids that are formed as part of the development of the sexual fruiting body and that will go through meiosis are from homokaryons and will therefore be homozygous, apart from any spontaneous mutations (Figure 1A).) The entire plate was then scraped and washed with 5 ml of a soap solution (water containing NaCl 0.8% and Tween-80 0.05%), collecting all nuclei present on the surface of the entire Petri dish. After vigorous vortexing of the mixture for 50 s, the number of nuclei was estimated via serial dilution and plate counts on CM medium supplemented with Triton X-100 (40 μl/l). The addition of Triton ensures that fungal colonies remain small, facilitating the accurate counting of up to 200 colonies per plate. The number of nuclei produced per unit time was calculated by multiplying the colony counts by the surface area of a colony after 5 days, calculated as $\pi*(MGR/2)^2$. This multiplication scales the total number of nuclei, as measured in the current assay in which all genotypes covered the same circular area, by the observed surface area covered by the genotype in 5 days of growth, as determined from the separate fitness (MGR) assay described above. These values were *ln* transformed before analysis.

Testing for FAS

To remove any environmental effects that could contribute to a negative relationship between fitness and sexual investment within genotypes, we calculated the residuals from a regression of the investment in sex on environment, thereby removing the main effects of environment and any of the effects of fitness that covary with it. These residuals were then used in independent regressions to calculate the slope of the relationship of investment in sex on fitness (MGR) for each genotype. A one-sample t test was then used to compare the mean of this distribution of slopes against the null hypothesis that the slope is zero (i.e., no FAS). This analysis preserves genotypes as the independent unit of replication. In the above analyses, sexual fruiting body counts were first transformed as *ln* (sexual fruiting body density +1) to meet the assumption of normality.

Supplemental Information

Supplemental Information includes two tables and can be found with this article online at doi:10.1016/j.cub.2010.05.060.

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